PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:	Davin C. Dillon *et al.*
Group Art Unit:	1637
Application No:	10/010,742
Filed:	November 30, 2001
For:	COMPOSITIONS AND METHODS FOR THE THERAPY AND DIAGNOSIS OF BREAST CANCER
Examiner:	Teresa E. Strzelecka, Ph.D.
Docket No.:	210121.491C7

DECLARATION OF DR. DAVIN DILLON, Ph.D.

Mail Stop Amendment
Commissioner for Patents
Washington, D.C. 20231

The undersigned, Dr. Davin Dillon, hereby declares:

1. I am a Scientist and Director, Product Management at Corixa Corporation, the assignee of the subject application, and a named inventor of the present invention.

2. I have reviewed the Office Action dated May 26, 2004 and in particular the rejections under 35 U.S.C. §§ 101 and 112, and am familiar with the instant application. I provide this Declaration to assist the Examiner in analyzing the claimed invention.

3. The following analyses and experiments were carried out under my supervision.

4. The specification as filed clearly states at page 103, lines 17-25 that the polynucleotide set forth in SEQ ID NO:52 was determined to be overexpressed in breast tumor tissue compared to other tissues tested by a visual analysis of microarray data. Further, using computer sequence analysis software available in the art and described in the specification, such as the DNAStar Seqman™ program, the specification states that SEQ ID NO:52 matches the two

template sequences of 228686.6 and 228686.8, the polynucleotide sequences of which are set forth in SEQ ID NOs:302 and 304. Further, using the DNAStar sequence analysis program or other commercially available sequence analysis programs, it is easily confirmed by the skilled artisan that SEQ ID NO:52 is 100% identical to the polynucleotide set forth in SEQ ID NO:305 as shown by the attached alignment in Figure 1. Based on this analysis, I conclude that SEQ ID NO:52 is a partial sequence of the polynucleotide set forth in SEQ ID NO:305 (also referred to as B854P) and, as such, the polynucleotide set forth in SEQ ID NO:305 would be expected to have the same expression profile as the polynucleotide set forth in SEQ ID NO:52.

5. To confirm that the polynucleotide set forth in SEQ ID NO:305 is overexpressed in breast tissue, real time PCR was carried out. The first-strand cDNA used in the quantitative real-time PCR was synthesized from 20 μg of total RNA that was treated with DNase I (Amplification Grade, Gibco BRL Life Technology, Gaithersburg, MD), using Superscript Reverse Transcriptase (RT) (Gibco BRL Life Technology, Gaithersburg, MD). Real-time PCR was performed with a GeneAmp™ 5700 sequence detection system (PE Biosystems, Foster City, CA). The 5700 system uses SYBR™ green, a fluorescent dye that only intercalates into double stranded DNA, and a set of gene-specific forward and reverse primers. The increase in fluorescence was monitored during the whole amplification process. The optimal concentration of primers was determined using a checkerboard approach and a pool of cDNAs from tumors was used in this process. The PCR reaction was performed in 25 μl volumes that included 2.5 μl of SYBR green buffer, 2 μl of cDNA template and 2.5 μl each of the forward and reverse primers for the gene of interest. The cDNAs used for quantitative real time PCR reactions were diluted 1:10 for each gene of interest and 1:100 for the β-actin control. Levels of mRNA were expressed relative to β-actin.

6. The real time PCR results showed that mRNA expression for B854P was present at a 10-100 fold higher level in approximately 30% of breast tumors as compared to a panel of normal tissues (see Figure 2).

7. Immunohistochemical (IHC) analysis was performed to determine B854P protein expression in breast cancer and normal tissues. IHC analysis was performed with the affinity purified anti-B854P polyclonal antibodies generated to the peptide sequences 1-4 shown below.

(1) VIQDRKESLKDKLKQDTTQKRRW, amino acid residues 260-282 of the B854P protein as set forth in SEQ ID NO:307.

(2) GHKEFYPVKEFEVYHKLMEKYPC, amino acid residues 56-78 of the B854P protein as set forth in SEQ ID NO:307.

(3) GRGLVTLDGSKWKKHRQIVKPGF, amino acid residues 122-144 of the B854P protein as set forth in SEQ ID NO:307.

(4) HQGSIQLDSTLDSYLKAVFNLSKI, amino acid residues 198-221 of the B854P protein as set forth in SEQ ID NO:307.

8. To generate the polyclonal antibodies, 400 micrograms of the combined peptides that were conjugated to KLH was combined with 100 micrograms of muramyldipeptide (MDP). Equal volume of Incomplete Freund's Adjuvant (IFA) was added and then mixed. Every four weeks animals were boosted with 100 micrograms of antigen mixed with an equal volume of IFA. Seven days following each boost the animal was bled. Sera was generated by incubating the blood at 4° C for 12-24 hours followed by centrifugation.

9. The polyclonal antisera was characterized as follows. Ninety-six well plates were coated with antigen by incubating with 50 microliters (typically 1 microgram) at 4° C for 20 hours. Two hundred and fifty microliters of BSA blocking buffer was added to the wells and incubated at room temperature (RT) for 2 hours. Plates were washed 6 times with PBS/0.01% Tween. Rabbit sera was diluted in PBS. Fifty microliters of diluted sera was added to each well and incubated at RT for 30 minutes. Plates were washed as described above before 50 microliters of goat anti-rabbit horse radish peroxidase (HRP) at a 1:10000 dilution was added and incubated at RT for 30 minutes. Plates were washed as described above and 100 microliters of TMB microwell Peroxidase Substrate was added to each well. Following a 15-minute incubation in the dark at room temperature the colorimetric reaction was stopped with 100 microliters of 1N H_2SO_4 and read immediately at 450 nm.

10. For IHC, paraffin-embedded formalin fixed tissue was sliced into 4-micron sections. Steam heat induced epitope retrieval (SHIER) in 0.1 M sodium citrate buffer (pH 6.0) was used for optimal staining conditions. Sections were incubated with 10% serum/PBS for 5 minutes. Primary antibody was added to each section for 25 minutes at indicated concentrations followed by a 25-minute incubation with an anti-rabbit biotinylated

antibody. Endogenous peroxidase activity was blocked by three 1.5-minute incubations with hydrogen peroxidase. The avidin biotin complex/horse radish peroxidase (ABC/HRP) system was used along with DAB chromogen to visualize antigen expression. Slides were counterstained with hematoxylin. As summarized in Table 1 below, 9/10 breast cancer samples were positive for B854P immunoreactivity as were 5/5 normal breast samples. Normal colon showed some reactivity over background whereas thyroid, liver and tonsil were negative.

TABLE 1: SUMMARY OF IHC ANALYSIS OF B854P EXPRESSION

Tissue Type	No. Tissues Positive/No. Tested
Breast Cancer	9/10
Normal Breast	5/5
Liver	0/1
Thyroid	0/1
Tonsil	0/1
Colon	1/1

11. Accordingly, these data confirm, as described in the specification as filed, that B854P has a breast-specific expression profile. Thus, this antigen can be used in any number of diagnostic and therapeutic applications. For example, overexpression of B854P in breast tumor tissue and normal breast tissue, as compared to other normal tissue types, *e.g.*, PBMCs, can be exploited diagnostically. In this case, the presence of metastatic breast tumor cells, for example in a sample taken from the circulation or liver, can be identified and/or confirmed by detecting expression of B854P in the sample, for example using RT-PCR or by a binding assay as described in the specification as filed. It should be noted that expression of the B854P protein in normal breast tissue does not preclude its use as a diagnostic indicator nor is it a concern with regard to therapeutic applications.

12. The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful, false

statements, and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

Davin Dillon, Ph.D.

July 12, 2004
Date

Figure 1

Seq1(1>379)	Seq2(1>1518)	Similarity	Gap	Gap	Consensus
491c7 seq 52	491c7 seq 305	Index	Number	Length	Length
(379<-1)	(619>997)	100.0	0	0	379

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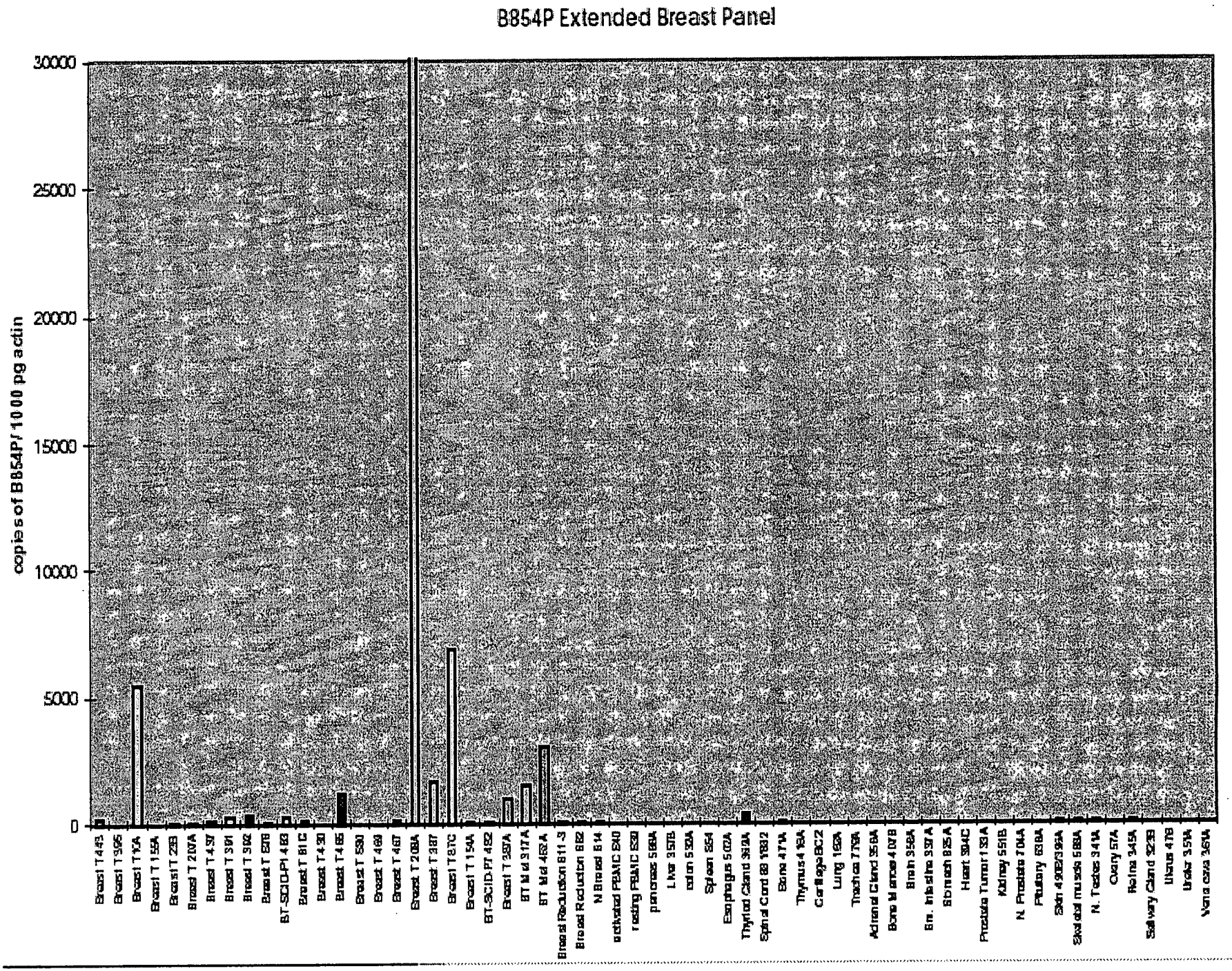
          ₣370       ₣360       ₣350       ₣340       ₣330       ₣320       ₣310
ACCCTGGACTCATACCTGAAAGCAGTGTTCAACCTTAGCAAAATCTCCAACCAGCGCATGAACAATTTTC
ACCCTGGACTCATACCTGAAAGCAGTGTTCAACCTTAGCAAAATCTCCAACCAGCGCATGAACAATTTTC
ACCCTGGACTCATACCTGAAAGCAGTGTTCAACCTTAGCAAAATCTCCAACCAGCGCATGAACAATTTTC
 ₺620      ₺630      ₺640      ₺650      ₺660      ₺670      ₺680
          ₣300       ₣290       ₣280       ₣270       ₣260       ₣250       ₣240
TACATCACAACGACCTGGTTTTCAAATTCAGCTCTCAAGGCCAAATCTTTTCTAAATTTAACCAAGAACT
TACATCACAACGACCTGGTTTTCAAATTCAGCTCTCAAGGCCAAATCTTTTCTAAATTTAACCAAGAACT
TACATCACAACGACCTGGTTTTCAAATTCAGCTCTCAAGGCCAAATCTTTTCTAAATTTAACCAAGAACT
 ₺690      ₺700      ₺710      ₺720      ₺730      ₺740      ₺750
          ₣230       ₣220       ₣210       ₣200       ₣190       ₣180       ₣170
TCATCAGTTCACAGAGAAAGTAATCCAGGACCGGAAGGAGTCTCTTAAGGATAAGCTAAAACAAGATACT
TCATCAGTTCACAGAGAAAGTAATCCAGGACCGGAAGGAGTCTCTTAAGGATAAGCTAAAACAAGATACT
TCATCAGTTCACAGAGAAAGTAATCCAGGACCGGAAGGAGTCTCTTAAGGATAAGCTAAAACAAGATACT
 ₺760      ₺770      ₺780      ₺790      ₺800      ₺810      ₺820
          ₣160       ₣150       ₣140       ₣130       ₣120       ₣110       ₣100
ACTCAGAAAAGGCGCTGGGATTTTCTGGACATACTTTTGAGTGCCAAAAGCGAAAACACCAAAGATTTCT
ACTCAGAAAAGGCGCTGGGATTTTCTGGACATACTTTTGAGTGCCAAAAGCGAAAACACCAAAGATTTCT
ACTCAGAAAAGGCGCTGGGATTTTCTGGACATACTTTTGAGTGCCAAAAGCGAAAACACCAAAGATTTCT
 ₺830      ₺840      ₺850      ₺860      ₺870      ₺880      ₺890
          ₣90        ₣80        ₣70        ₣60        ₣50        ₣40        ₣30
CTGAAGCAGATCTCCAGGCTGAAGTGAAAACGTTCATGTTTGCAGGACATGACACCACATCCAGTGCTAT
CTGAAGCAGATCTCCAGGCTGAAGTGAAAACGTTCATGTTTGCAGGACATGACACCACATCCAGTGCTAT
CTGAAGCAGATCTCCAGGCTGAAGTGAAAACGTTCATGTTTGCAGGACATGACACCACATCCAGTGCTAT
 ₺900      ₺910      ₺920      ₺930      ₺940      ₺950      ₺960
          ₣20        ₣10
CTCCTGGATCCTTTACTGCTTGGCAAAGT
CTCCTGGATCCTTTACTGCTTGGCAAAGT
CTCCTGGATCCTTTACTGCTTGGCAAAGT
 ₺970      ₺980      ₺990
```

Figure 2


B854P Extended Breast Panel
copies of B854P/1000 pg actin
30000
25000
20000
15000
10000
5000
0
Breast T 443
Breast T 305
Breast T 15A
Breast T 155A
Breast T 228
Breast T 20NA
Breast T 437
Breast T 391
Breast T 392
Breast T 576
BT-SCID-PT 483
Breast T 61C
Breast T 430
Breast T 485
Breast T 530
Breast T 469
Breast T 467
Breast T 208A
Breast T 387
Breast T 87C
Breast T 154A
BT-SCID-PT 452
Breast T 397A
BT Mid 917A
BT Mid 482A
Breast Reduction 811-3
Breast Reduction 882
N Breast 814
activated PBMC 840
resting PBMC 839
pancreas 568A
Liver 357B
colon 530A
Spleen 554
Esophagus 502A
Thyroid Gland 382A
Spinal Cord 88 1882
Bone 477A
Thymus 419A
Cartilage 802Z
Lung 162A
Trachea 779A
Adrenal Gland 356A
Bone Marrow 407B
Brain 358A
Sm. Intestine 397A
Stomach 825A
Heart 394C
Prostate Tumor 133A
Kidney 551B
N. Prostate 704A
Pituitary 638A
Skin 490B/399A
Skeletal muscle 588A
N. Testes 341A
Ovary 57A
Retina 345A
Salivary Gland 923B
Uterus 47B
Urethr 3.5A
Vena cava 369A

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2069–2076 (2004)

Cancer Diagnostics

Mammaglobin as a Novel Breast Cancer Biomarker: Multigene Reverse Transcription-PCR Assay and Sandwich ELISA

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Background: **The aim of this study was to examine the potential usefulness of a mammaglobin multigene reverse transcription-PCR (RT-PCR) assay and a mammaglobin sandwich ELISA as diagnostic tools in breast cancer.**

Methods: **We studied peripheral blood samples from 147 untreated Senegalese women with biopsy-confirmed breast cancer and gathered patient information regarding demographic, and clinical staging of disease. The samples were tested for mammaglobin and three breast cancer-associated gene transcripts by a multigene real-time RT-PCR assay and for serum mammaglobin protein by a sandwich ELISA assay.**

Results: **In 77% of the breast cancer blood samples, a positive signal was obtained in the multigene RT-PCR assay detecting mammaglobin and three complementary transcribed genes. Fifty samples from healthy female donors tested negative. Significant correlations were found between mammaglobin protein in serum, presence of mammaglobin mRNA-expressing cells in blood, stage of disease, and tumor size. Circulating mammaglobin protein was detected in 68% of the breast cancer sera, and was increased in 38% in comparison with a mixed control population. The RT-PCR assay and the ELISA for mammaglobin produced a combined sensitivity of 84% and specificity of 97%.**

Conclusion: **The ELISA and RT-PCR for mammaglobin and mammaglobin-producing cells could be valuable tools for diagnosis and prognosis of breast cancer.**

The identification of sensitive and specific biomarkers for the detection of circulating breast cancer cells and for staging of breast cancer may be of considerable importance for clinical management of breast cancer and could provide an important tool for researchers. Previous studies have demonstrated the usefulness of reverse transcription-PCR (RT-PCR)[5]-based detection of mammaglobin, a homolog of the rat prostatic binding protein component 3 *(1)* and a member of the uteroglobin/clara cell protein family (secretoglobins) *(2)*, for identification of disseminated breast cancer cells in blood, lymph nodes, and bone marrow *(3–5)*. Mammaglobin, which is almost exclusively expressed in breast epithelial cells *(6)*, is also overexpressed in a subset (70–80%) of primary and metastatic breast cancer tissues *(7)*.

We recently developed a panel of four complementary expressed genes, mammaglobin, B305D, γ-aminobutyrate type A receptor π-subunit (GABAπ), and B726P, to provide a panel with high sensitivity and specificity for detection of circulating breast cancer cells *(8, 9)*. In our previous study, we evaluated this multigene RT-PCR assay in 27 primary breast cancer tissues, 50 lymph nodes infiltrated with metastatic breast cancer, and 27 non-breast cancer lymph node specimens *(10)*. All primary breast tumors and metastatic breast cancer lymph nodes, but none of the control samples, demonstrated positive expression signals of mammaglobin, B305D, GABAπ,

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[5] Nonstandard abbreviations: RT-PCR, reverse transcription-PCR; GABAπ, γ-aminobutyrate type A receptor π-subunit; and CEA, carcinoembryonic antigen.

and/or B726P. In the present study we focus on transcript detection of this gene panel in peripheral blood.

Detection of circulating biomarkers is important for diagnosis, prognosis, and monitoring of breast cancer patients. In addition to the classic biomarker CA15.3, newer prognostic and diagnostic circulating markers such as kallikreins 5 and 14 *(11, 12)* have recently been reported. Although little is known about detection of circulating mammaglobin protein at present, previous studies have suggested that detection of mammaglobin protein has potential as a biomarker for breast cancer *(13, 14)*. Thus, in the present study we examined the relationship between peripheral blood-based detection of mammaglobin transcripts detected by a multigene RT-PCR assay and of mammaglobin protein concentrations in breast cancer patients and control individuals.

Materials and Methods

PATIENTS

Beginning in February 2001, Senegalese women presenting to the Oncology Service at the Dantec Hospital of the University of Dakar with masses that were clinically diagnosed as breast cancer, and who had not undergone previous biopsy, surgery, or therapy, were invited to enroll in this study. Written informed consent was obtained in compliance with the Human Subjects Institutional Review Boards of the University of Washington and the University of Dakar. A 10-mL sample of blood was collected into EDTA Vacutainer Tubes and was immediately sent to the laboratory for processing. After collection of blood, a physical examination was performed, and tissue samples were obtained from the main tumor mass by needle core biopsy. Two adjacent needle core biopsies were obtained; one was placed into formalin for routine histologic processing and the other in RNAlater reagent (Ambion) for molecular studies. Chest x-rays and ultrasounds were performed to stage disease. In total, 197 patients were enrolled, of whom 147 (75%) were found to have biopsy-confirmed breast cancer. Serum samples from 142 patients with pathology-confirmed breast cancer were available for ELISA studies, and peripheral blood samples from 84 patients were available for RT-PCR testing for circulating breast cancer cells. Control serum samples were collected from 53 Senegalese and 41 US women without breast cancer. Control blood samples for the RT-PCR assay were collected from 50 healthy female volunteers at Corixa in Seattle.

TUMOR CELL ENRICHMENT FROM PERIPHERAL BLOOD

In the laboratory, RosetteSep™ CD45 depletion cocktail for enrichment of circulating epithelial tumor cells (StemCell Technologies Inc.) was added (at a concentration of 50 μL/mL of whole blood) to the Vacutainer Tube, which was then incubated at room temperature for 20 min. The antibody-treated blood was then transferred to Accuspin System-Histopaque-1077 tubes (cat. no. A6929; Sigma Chemical Co.) and centrifuged for 10 min at 1000*g* to separate the human epithelial cells from the hematopoietic cells that were antibody cross-linked to erythrocytes. The cell layer was collected and washed once with phosphate-buffered saline, and cell pellets were resuspended with 1.5 mL of mRNA isolation lysis buffer (Roche) and stored in liquid nitrogen for shipment to Seattle. The breast cancer cell lines MDA-MB-415 (ATCC HTB-128) and BT-474 (ATCC HTB-20) were grown in DMEM (Gibco, Invitrogen) containing 100 mL/L fetal bovine serum (HyClone) at 37 °C in a 5% CO_2 atmosphere. Both cell lines were added to 5-mL aliquots of peripheral blood from a healthy donor at 0, 1, 10, and 100 cells/mL to establish detection limits of the assay.

RNA EXTRACTION AND cDNA SYNTHESIS

Needle core biopsies in RNAlater reagent were shipped to Seattle on liquid nitrogen. Tissue samples (10–30 mg each) were transferred into 1 mL of lysis buffer [Ambion Poly(A) Pure mRNA Purification Kit] and disrupted by agitation with 2 g of 1 mm zirconia beads (BioSpec Products Inc.) for 3 min in a MiniBeadBeater (MidWest Scientific) run on the highest setting. RNA was isolated according to the manufacturer's protocol and eluted with 60 μL of elution buffer. Reverse transcription was performed for 1 h at 42 °C with oligo(dT) primers and 10 μL of Superscript (Invitrogen) in a final volume of 150 μL.

For peripheral blood cell samples, the tumor cell-enriched blood cell lysates were shipped to Seattle on liquid nitrogen and processed according to the manufacturer's protocol (mRNA Isolation Kit; Roche). mRNA was eluted with 25 μL of nuclease-free H_2O and reverse-transcribed into cDNA by use of oligo(dT) primers (Gibco) and 8 μL of Superscript Reverse Transcriptase (Gibco) in a final volume of 120 μL.

MULTIGENE REAL-TIME RT-PCR

The specific primers and 6-carboxyfluorescein-labeled TaqMan® probes used to detect mRNA expression of mammaglobin, GABAπ, B305D, and B726P simultaneously are shown in Table 1. Primers were designed to cross intron–exon junctions to exclude genomic DNA from amplification. Expression was measured by quantitative real-time PCR with the ABI 7700 Prism™ sequence detection system (Applied Biosystems). Actin expression was measured in separate reactions as a quality control for blood cDNA samples. Specimens with actin expression <50 copies were excluded from analysis.

Fifty PCR cycles were performed with TaqMan 1000 Rxn PCR Core Reagents (part no. 430 4439; Applied Biosystems) and 0.0375 U/μL TaqGold, 1× Buffer A; 5 mM MgCl, 0.2 mM each of dCTP, dATP, and dGTP; 0.4 mM dUTP; 0.01 U/μL AmpErase UNG; 80 nL/μL glycerol; 0.5 nL/μL gelatin; and 0.1 nL/μL Tween 20. PCR conditions were 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, and 50 cycles 95 °C for 15 s, 60 °C for 1 min, and 68 °C for 1 min. Multigene copy numbers were calculated by use of a calibration curve constructed with

Table 1. Primers and probes for the multigene quantitative RT-PCR method.

Gene	Primers and probe[a]	Concentration	Amplicon size, bp
Mammaglobin	F:[b] TGCCATAGATGAATTGAAGGAATG	100 nM	89
	R: TGTCATATATTAATTGCATAAACACCTCA	100 nM	
	P: TCTTAACCAAACGGATGAAACTCTGAGCAATG	4 pmol	
GABAπ	F: CAATTTTGGTGGAGAACCCG	300 nM	137
	R: GCTGTCGGAGGTATATGGTG	50 nM	
	P: CATTTCAGAGAGTAACATGGACTACACA	4 pmol	
B305D	F: TCTGATAAAGGCCGTACAATG	300 nM	239
	R: TCACGACTTGCTGTTTTTGCTC	50 nM	
	P: ATCAAAAAACAAGCATGGCCTCACACCACT	4 pmol	
B726P	F: GCAAGTGCCAATGATCAGAGG	100 nM	110
	R: ATATAGACTCAGGTATACACACT	100 nM	
	P: TCCCATCAGAATCCAAACAAGAGGAAGATG	4 pmol	
β-Actin	F: ACTGGAACGGTGAAGGTGACA	300 nM	
	R: CGGCCACATTGTGAACTTTG	300 nM	
	P: CAGTCGGTTGGAGCGAGCATCCC	4 pmol	

[a] All sequences shown in the 5'→3' direction.
[b] F, forward primer; R, reverse primer; P, probe.

TaqMan SDS analysis software from serial dilutions of four plasmids containing target gene cDNA sequences. Final copy numbers were determined as medians of triplicate reactions. Triplicate reactions were pooled, and a 20-μL aliquot was separated by agarose electrophoresis using a 4% E-Gel (Invitrogen). Gene identities were determined according to amplicon size.

MAMMAGLOBIN ELISA

Antibodies were generated against purified native mammaglobin protein complex *(15)*. We coated 96-well microtiter plates overnight at 4 °C with the monoclonal antibody RO48 at 200 ng/well. The wells were then blocked by incubation with 50 g/L nonfat milk in phosphate-buffered saline for 2 h at room temperature. For constructing the calibration curve, different dilutions of purified native mammaglobin protein complex were prepared in normal human serum. Biotinylated RO28 (1 mg/L) was added and incubated at room temperature for 1 h. Plates were washed, and streptavidin-horseradish peroxidase was added and incubated for 30 min. Tetramethylbenzidine substrate was added for 15 min before the reaction was stopped with 0.05 mol/L sulfuric acid, and the absorbance was read at 450 nm. Each specimen was run in duplicate, and CVs were <10%.

STATISTICAL ANALYSIS

Two-sided Mantel–Haenszel χ^2 or Fisher exact tests were performed to assess univariate associations between gene detection and the demographic and clinical characteristics of the breast cancer patients or their disease. Demographic characteristics evaluated included age, gravidity, birth control use, menopausal status, history of hormonal therapy, alcohol and tobacco use, and history of obesity. Each breast cancer was characterized by tumor size, lymph node involvement, and metastasis and was subsequently staged by standard breast cancer staging methods. Associations with ordered categorical factors were tested using Mantel–Haenszel tests for trend, and the Student *t*-test or ANOVA was used to compare groups with respect to continuous variables. Data analyses were performed with SAS 8.2 for Windows (SAS Institute). A ROC curve was used to determine the optimum mammaglobin protein concentrations predictive of breast cancer (>1.71 μg/L). The optimum threshold was determined as the predictive probability such that both sensitivity and specificity were simultaneously maximized *(16)*.

Results

PATIENT CHARACTERISTICS

Histologic and cytologic review of biopsies confirmed the presence of breast cancer in 147 Senegalese patients. The mean age of the patients was 47.7 years (range, 13–77 years). Approximately one half (47%) were menopausal, and only 10 currently used any form of hormonal contraception. Mean gravidity was 5.8 (range, 0–19), and mean age of first pregnancy was 19.4 years. Few (3%) had any history of hormone replacement therapy, and <1% reported tobacco or alcohol use. Eleven women (7%) had a history of obesity. Most of these women had advanced disease and large tumors, with only 33% of tumors being 5 cm or less in size, whereas 43% were 10 cm or greater (Table 2). Evidence of nodal involvement was present in 92% of cases, and the vast majority (80%) of the cancers were stage III and higher.

GENE TRANSCRIPTS IN BLOOD AND TISSUE

To determine the sensitivity of the multigene RT-PCR assay, we added the breast cancer cell lines MDA-MB-415 and BT-474 to peripheral blood aliquots from a healthy volunteer (Fig. 1). Although no formal determination was made of the assay's detection limit or limit of quantifica-

Table 2. Clinical characteristics of tumors in women with confirmed breast cancer in Senegal (n = 147).

Characteristic	n (% with characteristic)
Tumor size,[a] cm	
2 cm	5 (4%)
3–5 cm	42 (29%)
6–9 cm	35 (24%)
≥10 cm	62 (43%)
Tumor attached and spread to pectoral lymph nodes[a]	40 (27%)
Lymph nodes[a]	
N0	12 (8%)
N1	73 (50%)
N2/3	61 (42%)
Clinical stage[a]	
I	1 (1%)
IIa	6 (4%)
IIb	24 (17%)
IIIa	19 (13%)
IIIb	42 (29%)
IV	53 (37%)

[a] Missing data: tumor size (n = 3); tumor attachment (n = 1); lymph nodes (n = 1); clinical stage (n = 2).

tion, multigene real-time expression signal was detected in this study at 1 cell/mL of blood for both cell lines. Similarly, mammaglobin real-time RT-PCR detected an expression signal at 1 cell/mL for MDA-MB-415 and at 100 cells/mL for BT-474.

Gene expression analysis of cells collected from blood of 84 women with breast cancer and 50 healthy female volunteers was performed with the multigene RT-PCR assay. Mammaglobin expression was detected in 61%, GABAπ in 18%, B726P in 5%, and B305D in 8% of the blood samples. Overall, expression of one or more of these genes was present in 77% of samples from women with confirmed breast cancer but none of the 50 samples from women without cancer (Table 3).

We next examined the expression frequency of the four genes in corresponding breast cancer biopsies from 46 patients. Mammaglobin expression was detected in 85% (39 of 46) of cases (Table 3). GABAπ was detected in 52% of the tumors, but only when mammaglobin was also present. In two other cases, biopsy samples were positive for B726P or B305D, but not for mammaglobin. Thus, 89% of tissues examined expressed at least one of the studied genes.

ASSOCIATIONS BETWEEN GENE TRANSCRIPTS AND TUMOR CHARACTERISTICS

We next examined the associations between detection of gene transcripts in peripheral blood and tumor characteristics (Table 4). Overall, among women with biopsy-confirmed breast cancer, detection in peripheral blood of any of the four genes included in the multigene assay was not significantly associated with increasing tumor size ($P = 0.4$). Likewise, detection of mammaglobin transcripts alone was not associated with tumor size ($P = 0.4$). However, detection of GABAπ alone in peripheral blood

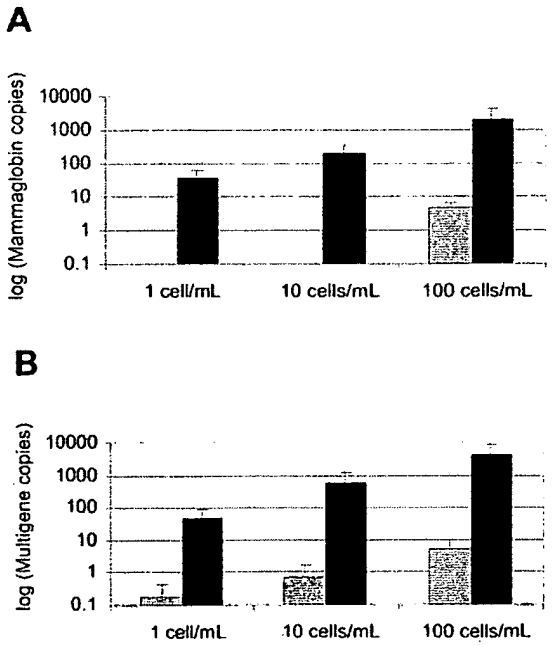

Fig. 1. Results of addition experiment in which the breast cancer cell lines MDA-MB-415 (■) and BT-474 (▨) were added to peripheral blood from a healthy donor.

The multigene RT-PCR assay detected expression signals at 1 cell/mL of peripheral blood for both cell lines (*B*). The detection limit for the mammaglobin single-gene RT-PCR assay was 1 cell/mL for MDA-MB-415 and 100 cell/mL for BT-474 (*A*). Both assays did not detect a signal in the 0 cells/mL control (data not shown).

Table 3. Detection of mammaglobin protein in sera and gene expression in tissue and blood from women with confirmed breast cancer in Senegal.

Assay	n (%)[b]
Gene expression[a]	
Tissue (n = 46)	
Mammaglobin	39 (85%)
GABAπ	24 (52%)
B726P	3 (7%)
B305D	3 (7%)
Multigene assay (any gene)	41 (89%)
Blood (n = 84)	
Mammaglobin	51 (61%)
GABAπ	15 (18%)
B726P	4 (5%)
B305D	7 (8%)
Multigene assay (any gene)	65 (77%)
Serum mammaglobin protein (n = 142)	
Any detected (>0 μg/L)	99 (70%)
Positive (>1.71 μg/L)	54 (38%)

[a] No gene transcripts were detected in blood samples from 50 healthy female volunteers.

[b] %, percentage of women in whom gene expression or serum protein was detected.

Table 4. Detection of breast cancer markers by clinical tumor characteristics.

	MG[a] PCR (n = 84)	GABAπ PCR (n = 84)	B726 PCR (n = 84)	B305 PCR (n = 84)	Multigene PCR (n = 84)	MG protein >1.71 μg/L (n = 142)
	51 (61.7%)	15 (17.9%)	4 (4.8%)	7 (8.3%)	65 (77.4%)	54 (38%)
Tumor size[b]						
2–5 cm	17/30 (57%)	0/30 (0%)	2/30 (7%)	2/30 (7%)	21/30 (70%)	14/47 (30%)
6–9 cm	16/22 (73%)	3/22 (14%)	1/22 (5%)	1/22 (5%)	19/22 (86%)	12/31 (39%)
≥10 cm	17/30 (57%)	12/30 (40%)	1/30 (3%)	4/30 (13%)	24/30 (80%)	28/61 (46%)
Lymph nodes[b]						
LN0	2/10 (20%)	1/10 (10%)	2/10 (20%)	1/10 (10%)	6/10 (60%)	6/12 (50%)
LN1	28/42 (67%)	4/42 (10%)	1/42 (2%)	1/42 (2%)	32/42 (76%)	26/69 (38%)
LN2/3	20/31 (65%)	9/31 (29%)	0/31 (0%)	5/31 (16%)	26/31 (84%)	22/60 (37%)
Clinical stage[b]						
I	1/1 (100%)	0/1 (0%)	0/1 (0%)	0/1 (0%)	1/1 (100%)	0/1 (0%)
IIa	1/4 (25%)	0/4 (0%)	0/4 (0%)	0/4 (0%)	1/4 (25%)	3/6 (50%)
IIb	9/15 (60%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	9/15 (60%)	6/24 (25%)
IIIa	10/14 (71%)	1/14 (7%)	1/14 (7%)	1/14 (7%)	13/14 (93%)	5/16 (31%)
IIIb	13/19 (68%)	4/19 (21%)	0/19 (0%)	0/19 (0%)	14/19 (74%)	14/41 (34%)
IV	16/30 (53%)	9/30 (30%)	2/30 (7%)	6/30 (20%)	26/30 (87%)	25/52 (48%)

[a] MG, mammaglobin.
[b] Missing data: tumor size (n = 2 for PCR; n = 3 for protein); lymph nodes (n = 1); clinical stage (n = 1 for PCR; n = 2 for protein).

was associated with tumor size (P <0.001, test for trend) with no expression in patients with small tumors (2–5 cm; n = 30), expression in 14% of medium tumors (6–9 cm; n = 22), and expression in 40% of the largest tumors (≥10 cm; n = 30). In addition, GABAπ was not detected in stage I, II, or IIIA cancers but was expressed in blood samples from 29% of those with stage IIIB or IV disease (P = 0.004, test for trend). Mammaglobin (P = 0.07) and GABAπ (P = 0.05) were each marginally associated with increased nodal involvement; only 20% of those with N0 disease were positive for mammaglobin compared with 60–70% of those with N1 or N2 disease. Similarly, 10% of women with N0 or N1 disease, compared with 29% of women with N2 disease, had GABAπ detected in blood samples. Overall, detection of the four gene transcripts was associated with stage of disease (P = 0.03), being present in 53% of those with stage II, 82% of those with stage III, and 87% of those with stage IV breast cancer.

Demographic and behavioral characteristics were also examined in association with detection of gene transcripts in peripheral blood. Detection of mammaglobin or other transcripts did not vary significantly with the age or gravidity of the patient; however, detection of GABAπ transcripts was inversely associated with menopausal status, as 28% of premenopausal compared with 9% of postmenopausal women with breast cancer had GABAπ transcripts detected in peripheral blood (P = 0.02).

ASSOCIATIONS BETWEEN SERUM MAMMAGLOBIN AND TUMOR CHARACTERISTICS

Circulating mammaglobin protein was evaluated in sera from 142 women with confirmed breast cancer and was detected in 70% of the samples. In addition, sera from 53 Senegalese and 38 US women without breast cancer were available, and ROC curve analysis was used to determine the optimum (highest sum of sensitivity and specificity) mammaglobin protein concentration predictive of breast cancer. A concentration of 1.71 μg/L was established for positivity, giving a breast cancer-specific mammaglobin protein concentration that was positive in 38% of the 142 cancer sera samples but only in 3% of the 91 control sera.

Among the 142 women with confirmed breast cancer, mammaglobin serum protein >1.71 μg/L was marginally associated with increasing tumor size (P = 0.09, test for trend), but not with lymph node involvement (P = 0.5). In addition, mammaglobin protein >1.71 μg/L was marginally associated with increasing clinical stage of disease (P = 0.10, test for trend), with only 29% of samples from women with stage I or II cancer being positive for mammaglobin protein compared with 31% of those with stage IIIA, 34% of those with stage IIIB, and 48% of those with stage IV disease (Table 4). None of the US controls and only three of the Senegal control sera had increased serum mammaglobin.

Interestingly, among those with mammaglobin serum protein above the concentration selected as the upper limit of normal (>1.71 μg/L; n = 54), mammaglobin serum concentration was predictive of disease severity. Mean natural log-transformed mammaglobin serum concentrations in clinical stages I to IIIB were all between 0.9 and 1.4 μg/L, but increased to 2.3 μg/L in stage IV disease (P = 0.02, ANOVA; Fig. 2). Similarly, mammaglobin serum concentrations were strongly associated with increasing tumor size (P = 0.001, ANOVA): the mean natural log-transformed mammaglobin serum concentration in tumors <10 cm was 1.2 μg/L compared with 2.3 μg/L in tumors 10 cm or larger.

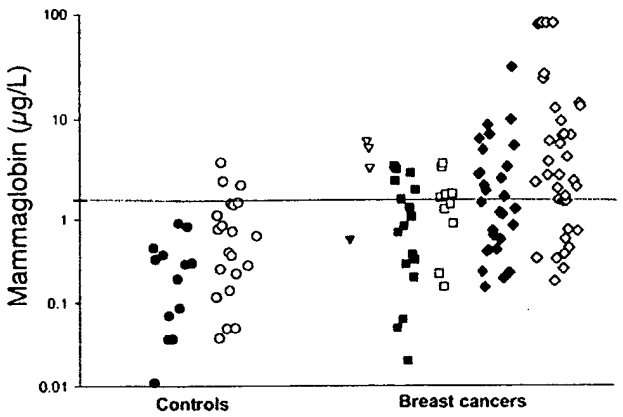

Fig. 2. Serum mammaglobin concentrations in control and breast cancer specimens.

Concentration of mammaglobin complex in serum specimens from 41 control US donors (●), 53 control Senegalese donors (○), and 142 Senegal breast cancer patients with different stages of disease (▼, stage I; ▽, stage IIA; ■, stage IIB; □, stage IIIA; ◆, stage IIIB; ◇, stage IV). A cutoff value of 1.71 μg/L was established by ROC analysis.

DETECTION OF MAMMAGLOBIN PROTEIN AND/OR GENE TRANSCRIPTS IN PERIPHERAL BLOOD SAMPLES

Blood samples from 79 women with breast cancer were analyzed by the multigene RT-PCR assay and for mammaglobin protein by ELISA. In these women, 84% had either increased mammaglobin protein or detectable multigene transcripts. Three patient samples had increased mammaglobin protein concentrations but were negative for mammaglobin mRNA, whereas 17 samples were positive for mammaglobin mRNA but mammaglobin serum protein was <1.71 μg/L. More than one half (59.5%) of the breast cancer blood samples had mammaglobin transcripts detected, 14 more samples had gene transcripts from one or more of B305D, GABAπ, and B726P detected, and an additional 5 samples had increased mammaglobin protein as quantified by ELISA.

The serum mammaglobin protein concentration was also associated with the detection of mammaglobin transcripts in blood by RT-PCR. In mammaglobin PCR-negative blood samples, the mean ELISA value for circulating protein was 1.8 μg/L compared with a mean ELISA value of 18.5 μg/L in mammaglobin PCR-positive samples ($P = 0.01$, t-test). Serum mammaglobin concentrations were also correlated to the log(copy numbers) of the multigene RT-PCR assay ($P = 0.02$).

Discussion

Development of biomarkers for detection and staging of breast cancer is of importance for clinical management of the disease. Several molecules, including carcinoembryonic antigen (CEA) and mucin-type markers (e.g., the MUC-1 gene and its glycoprotein antigens CA15.3 and CA27.29), have been used as biomarkers for metastatic breast cancer. Before-treatment sensitivities of the commonly used circulating tumor markers for breast cancer, CEA and CA15.3, have been reported as 12% *(17)* with an increase to 40% in breast cancer patients with recurrence. Several studies have reported the detection of circulating tumor cells by cytokeratins, CEA, and MUC-1 RT-PCR *(3, 18)*, but application of these assays has been hampered by lack of specificity *(19–21)*.

In this study, we examined the expression of mammaglobin, B305D, GABAπ, and B726P and the increase in mammaglobin protein concentrations in peripheral blood samples from breast cancer patients. For a cell-addition experiment in blood of a healthy volunteer, two breast cancer cell lines were used. MDA-MB-415 cells express ~100-fold higher concentrations of mammaglobin mRNA than BT-474 (data not shown). The multigene RT-PCR was able to detect both cell lines at 1 cell/mL of peripheral blood. The mammaglobin single RT-PCR assay could also detect the MDA-MB-415 cell line at 1 cell/mL, but the lower detection limit for BT-474 cells was 100 cells/mL. These results demonstrate a lower detection limit for the multigene RT-PCR, in particular for breast tumor cells with low mammaglobin gene expression.

The study population consisted of a large number of women with untreated breast cancer, almost all of whom had breast cancer that had already metastasized to regional lymph nodes. Given this, it was anticipated that these women had a high likelihood of having circulating tumor cells present. Moreover, the study population evaluated is probably not representative of newly diagnosed breast cancer patients in the US. Nonetheless, using a single 10-mL blood sample obtained before physical examination and biopsy, we detected at least one of the four breast cancer-associated transcripts and/or increased mammaglobin protein in 84% of women with breast cancer. Analysis of multiple samples collected over a 24-h period, as required for optimal detection of bacteremia by blood culture, might have boosted sensitivity even further.

Although mammaglobin tissue expression has been shown in ~80% of breast cancers, previous studies using blood samples from patients reported detection of mammaglobin transcript in 25–54% of those with and in 10–25% of patients without metastatic breast cancer *(3, 18, 22–26)*. In this study, we found mammaglobin transcripts in 61% of single blood samples from breast cancer patients. This increased rate of detection may be related to the fact that the patients examined were untreated, whereas many of the women examined in previous studies had undergone chemotherapy. Treatment may lower the number of detected circulating tumor cells. As mentioned above, because mammaglobin is not expressed in all breast cancers, we developed and recently reported on three complementary expressed genes that, when used in combination with mammaglobin, provided increased sensitivity for identification of disseminated breast tumor cells in lymph node specimens *(10)*. In the present study, the addition of these three transcripts increased blood-based detection of circulating cells from 61% to 77% of women with breast cancer. It is possible

that obtaining additional samples may further increase the number of patients positive for breast cancer.

The sensitivity of the multigene assay was increased with increasing cancer stage, but detection of the mammaglobin transcript alone was only marginally associated with increased nodal involvement and not with other tumor or patient characteristics. Our findings agree with a recent study by Lin et al. *(27)*, which evaluated the correlation between mammaglobin expression in peripheral blood and known prognostic factors for breast cancer patients. Whereas mammaglobin mRNA expression was frequently shown to be increased in patients with unfavorable prognostic factors (tumor size and disease stage), no significant differences could be confirmed. The same group also reported that mammaglobin mRNA detection combined with CEA or CA15.3 increased the sensitivity from 54% of 33 metastatic breast cancer patients to 81% and 90%, respectively, suggesting that mammaglobin mRNA may be a useful adjunct to existing serum markers.

We found that GABAπ transcripts were more often present in breast cancer patients with larger tumors, nodal involvement, and advanced overall tumor stage. Interestingly, GABAπ expression was higher in women who were premenopausal. These findings demonstrate a possible application of this marker to monitor disease progression and treatment efficacy in premenopausal patients in particular. Surprisingly, expression of B726P and B305D was detected only in small subsets of breast cancer blood specimens (5% and 8%, respectively). To confirm the expression of these target genes in the primary breast tumors, we analyzed biopsy tissue samples in a subset ($n = 47$) of patients. The percentages of tumors expressing mammaglobin (85%) and GABAπ (53%) were consistent with earlier findings *(8, 9)*, but B726P and B305D were expressed in only 6% of the tumor biopsies tested. Previously, we reported B726P and B305D overexpression in 40–50% and 60–70% of primary and metastatic breast cancer specimens, respectively *(10)*, which has been confirmed by others for B726P *(28)*. Specific characteristics of the breast tumors or patients (e.g., median age, later stage, ethnicity) could be the reason for a different gene expression profile. This would argue that B726P and B305D might exhibit different detection sensitivities in other patient populations. We are currently analyzing samples from US breast cancer patients with different ethnicities, age groups, and early stages of disease to confirm the application of this assay and its components.

Increasing serum concentrations of mammaglobin protein were associated with increasing clinical stage and tumor size. In addition, we found a significant correlation between mammaglobin serum protein and mammaglobin expression detected in the blood. Whereas circulating mammaglobin protein was detected in 70% of the samples from breast cancer patients, only 38% were considered increased compared with the control group. The cutoff value of 1.71 μg/L was established by use of a Senegalese and a US cohort of normal sera samples. The mean mammaglobin concentration in the Senegal normal sera (0.37 μg/L) was significantly higher than the mean concentration in US samples (0.10 μg/L). This finding might reflect a population difference regarding parity, nursing, and undiagnosed benign or malignant breast diseases. All of these conditions could affect the amount of mammaglobin protein released into the blood stream because mammaglobin expression is associated with mammary ductal proliferation *(29)*. Detailed studies in different healthy populations need to be performed to identify conditions that could influence the serum mammaglobin protein concentration. Circulating mammaglobin protein may also be a good marker to monitor treatment and detect disease relapse. Moreover, the detection of serum mammaglobin protein could be combined with other markers, e.g., CEA, CA15.3, and circulating antibodies against tumor-specific epitopes, to further increase detection sensitivity.

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Breast Cancer and HER-2/neu: Diagnostic Tools for Targeted Therapy

Technology Spotlight

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With over 200,000 new cases and 40,000 deaths per year, breast cancer undoubtedly qualifies as one of America's most serious health problems. Recently, efforts to develop early detection methods and more effective therapy have made important progress. This is particularly true at the molecular level, where discoveries on protein expression have translated into new diagnostic tests and targeted therapies. An example is the finding that tumors expressing estrogen receptors (so called ER+ tumors) tend to respond to tamoxifen, a selective estrogen receptor modulator. Conversely, ER- tumors tend to respond better to chemotherapy and not to tamoxifen. Another revolutionary example is that of Her-2/neu protein (also called c-*erb*B2), which is overexpressed in approximately 25-30% of breast cancers. With herceptin (trastuzumab) and other preferential medications available to treat these patients, the need has emerged for routine laboratory methods to detect Her-2/neu overexpression. A wide array of tools has arrived to fulfill this purpose.

Human cells normally carry and express two copies of Her-2/neu, which is located on chromosome 17 and encodes a cell-surface receptor for epidermal growth factor. Like many growth factor receptors, Her-2/neu functions as a tyrosine kinase and moves cells forward on the cell cycle, which involves DNA replication and cell division. Her-2/neu therefore falls into the class of proto-oncogenes. A key discovery is that some 25-30% of breast cancers carry amplifications (multiple copies) of the Her-2/neu gene, which lead to overexpression of Her-2/neu protein. The over-abundance of Her-2/neu receptor accelerates the cell cycle and causes cells to divide more rapidly and haphazardly. This explain why Her-2/neu overexpression is associated with more rapid tumor growth and a worse prognosis.

The real value of the Her-2/neu discovery, and one that helps to counter the poor prognosis of Her-2/neu positive tumors, is that specific therapies such as adriamycin have proven more effective for patients with these tumors. The most celebrated medication, however, is probably Herceptin, (trastuzumab) a chimerized human-mouse monoclonal antibody that binds an exposed portion of the Her-2/neu receptor on the cell surface. Herceptin has been shown to shrink and even eliminate some tumors. Compared to chemotherapy alone, the addition of herceptin reduces tumor recurrence by half. Herceptin has thus earned a place as first-line therapy for qualified patients.

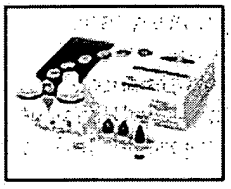
FDA-approved kits use one of three methods to quantify Her-2/neu overexpression in breast tumors. The first is immunohistochemistry (IHC), which involves a semiquantitative, microscopic assessment of antibody reactivity on a fixed tissue sample. Studies have shown that when specimens are carefully handled, IHC results correlate well with Her-2/neu copy number. The test uses a conventional microscope, can be automated, and has a relatively low cost. Some challenges of IHC include consistent tissue preparation and control assays. Commercially available and FDA-approved IHC kits include the InSite Her-2/neu kit (BioGenex), available in both manual and automated forms. The automated form is compatible with the BioGenex i6000 system. Other tests, which can also be automated, are the HercepTest (Dako Corporation) and PATHWAY (Ventana Medical Systems). The Ventana

PATHWAY offers an integrated system of IHC plus a reflex FISH assay (see below).

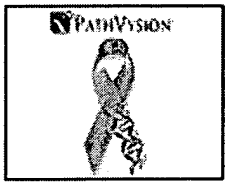
Fluorescent in situ hybridization (FISH) involves the hybridization of specific DNA probes, which are visible on a fluorescent microscope. FISH is compatible with all kinds of tissue, whether fixed or not. Like IHC, FISH is also amenable to automation. A particular benefit of FISH is that it contains an internal control: a chromosome 17 probe not associated with Her-2/neu. An excess of Her-2/neu probe fluorescence over control probe fluorescence indicates Her-2/neu amplification. Challenges of FISH include higher cost and the need for a fluorescent microscope. One commercially available kit is the Ventana INFORM, which works in concert with the IHC system mentioned above. Other FDA-approved kits are PathVysion (Abbott-Vysis) and the Her-2 FISH PharmDx kit (Dako USA).

ELISA is also available to quantify Her-2/neu expression. In contrast to IHC and FISH, ELISA can detect Her-2/neu as a serum marker. This bypasses the need for a tissue specimen and may allow earlier Her-2/neu detection. The Her-2/neu ELISA (Bayer Diagnostics Oncogene Science) is a sandwich-type immunoassay approved to monitor patients with known metastatic breast cancer.

With other methods such as RT-PCR on the horizon, one can expect further evolution of the diagnostic and therapeutic landscape. Along with it comes the promise of similar solutions for other cancer patients.



» **InSite™ Her-2/neu Kit - BioGenex Laboratories**
The Her-2/neu protein is a 185 kD trans-membrane glycoprotein associated with tyrosine kinase activity. Approximately 20-30% cases of breast cancer show an amplification and/or over-expression of Her-2/neu in tumor cells. Since the introduction of Herceptin® as a targeted therapy for breast cancer, the clinical testing of Her-2/neu in breast carcinoma has become very important in patient care.



» **PathVysion® HER-2 DNA Probe Kit - Vysis, Inc.**
PathVysion is the only HER-2 assessment test approved by the U.S Food and Drug Administration (FDA) for three claims: prognosis, adriamycin-based chemotherapy selection and Herceptin® (Trastuzumab) monoclonal antibody therapy selection. PathVysion is the only FISH-based DNA probe HER-2 test listed in the Herceptin® package insert as an appropriate test to aid in the selection of patients for Herceptin® therapy.

» **HER2 FISH pharmDx™ Kit - DakoCytomation California Inc.**
HER2 FISH pharmDx™ Kit is a direct fluorescence in situ hybridization (FISH) assay designed to quantitatively determine HER2 gene amplification in formalin-fixed, paraffin-embedded breast cancer tissue specimens. Gene amplification is determined from the ratio between the number of signals from the hybridization of the HER2 gene probe (red signals) and the number of signals from the hybridization of the reference chromosome 17 probe (green signals).

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